MnESI-MS Platform for Sensitive Analysis of Oligonucleotides

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Keywords

Microflow-nanospray ESI-MS (MnESI-MS), Ion source, M3 emitter, Multinozzle, Oligonucleotides, ASO, siRNA, Ion-pairing reagent, Quantitation, Microflow LC-MS, High-flow LC-MS, Flow Splitting

Goal

To establish a microflow-nanospray ESI-MS (MnESI-MS) platform for liquid chromatography-mass spectrometry (LC-MS) analysis of oligonucleotides, that achieves high sensitivity and high robustness, using Newomics® MnESI sources, M3 multinozzle emitters, and flow splitting kits (as needed for high-flow HPLC/UPLC systems).

Introduction

Oligonucleotide therapeutics including single-stranded antisense oligonucleotides (ASOs) and double-stranded siRNAs are rapidly growing. Compared to traditional hybridization-based methods such as ELISA and PCR, mass spectrometry (MS) analysis of oligonucleotide is more specific because it can discriminate and quantify not only the full-length products, but also their impurities and metabolites. High-flow ion-pairing reversed-phase chromatography has become the LC method of choice for oligonucleotide analysis [1]. However, current LC-MS methods have the challenges of low sensitivity for negative ionization mode, and high contamination to the MS because of the ion-pairing reagents required for LC separation of oligos. The Newomics award-winning silicon multinozzle emitters (M3 emitters) split the incoming microflow eluent into multiple nanoflows at each nozzle, thereby significantly enhancing the ionization efficiency and reducing the matrix effects for ESI-MS ([2-5] and Newomics Application Notes [6]). In this Application Note, we demonstrate a new microflow-nanospray ESI-MS platform for LC-MS analysis of a 21 mer DNA oligonucleotide. Compared to the high-flow LC-MS, we obtained over 30-fold improvement in sensitivity, with a CV of less than 5%. Our MnESI-MS platform is amenable for a microflow LC system, as well as a high-flow LC system when interfaced with a Newomics® flow splitting kit. Our platform for oligonucleotide analysis increases the sensitivity through increased ionization efficiency at the low flow rate, as well as reduces the MS contamination because of less ion-pairing reagents entering the mass spectrometers.

Methods

1. Reagent and sample preparation

The 21 mer DNA oligo CAG TCG ATT GTA CTG TAC TTA was synthesized and desalted at Integrated DNA Technologies. Ethylenediaminetetraacetic acid solution (EDTA, Cat. # 03690-100ML), HPLC-grade water and methanol were purchased from Sigma-Aldrich (St. Louis, MO). LC-MS grade Triethylamine (TEA) was purchased from RICCA chemical company (Cat. # RMB11205), and 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) was purchased from Honeywell Fluka (Cat. # 42060-50ML). DNA oligo was diluted in 1mM EDTA at 10 ng/µl before LC-MS analysis.

2. MnESI-MS platform for LC-MS analysis

10 ng of 21 mer oligo DNA was injected using a 1 µl full loop injection method. The microflow LC was performed with a BEH C18 column (Waters Cat. # 186009257). The high-flow LC was performed with an XBridge Oligonucleotide Premier C18 column (Waters Cat. # 186009836). Both columns have the same 130 Å pore size of C18 beads. The LC gradients were listed in **Table 1** and **Table 2**, respectively. Mobile phase A was 1.6 mM TEA and 20 mM HFIP; and Mobile phase B was 1.6 mM TEA and 20 mM HFIP in 50% methanol.

Table 1. LC gradient for microflow MnESI at 5 µl/min.

Time (min)	% B
0	10
3.0	70
3.1	99
4.1	99
4.2	10
7.0	10

Table 2. LC gradient for high-flow HESI at 250 µl/min.

Time (min)	% B
0	10
3.0	50
3.1	90
4.1	90
4.2	10
7.0	10

Mass spectra were acquired on a Thermo Fisher Orbitrap Q Exactive Plus mass spectrometer interfaced with an UltiMate 3000 RSLCnano UPLC system (Thermo Fisher Scientific). MnESI ion source was either directly connected to the microflow LC column or to a high-flow column through a Newomics® flow splitter.

We performed comparison of the LC-MS analysis among two platforms. The LC and MS source conditions were listed in **Table 3**. The following emitters were used in our studies: a Thermo Fisher high-flow needle insert assembly (HESI high-flow, single nozzle, 100 µm ID, catalog # Opton 53010), and a Newomics® M3 emitter (8 nozzles, 10 µm ID, catalog # E8N10MU01).

Table 3. LC and MS ion source conditions for the two platforms.

Platform	LC-MnESI-MS	High-flow LC-MS
Flow Rate (µl/min)	5	250
Column IDXL (mm)	0.3X50	2.1X50
Column Temperature (°C)	60	60
Ion Source	Newomics MnESI Source	Ion Max Source
Emitter/Sprayer	M3 emitter (10 µm ID, 8-nozzle)	HESI probe (100 µm ID)
Emitter/Probe Position	3.0 mm from ion transfer tube	Position B
Spray Voltage (kV)	2.7	2.8
Gas Flow	0	25 units sheath gas, 10 units AUX gas (320 °C)
Capillary Temperature	275 °C	300 °C

The MS acquisition parameters for the two platforms were the same and listed in **Table 4**.

Table 4. MS acquisition parameters.

Full MS scan range m/z	400-2,000
Resolution	140,000
Polarity	Negative
AGC target	1.0 X 10e6
Max injection time	100 ms
Microscans	1
S-lens RF level (%)	60

3. Data Analysis

Data were analyzed using Xcalibur software and Biopharma Finder 2.0 software (Thermo Fisher Scientific). Charge deconvolution was processed by Biopharma Finder 2.0 with the Xtract function.

Results and Discussion

1. Optimization of microflow LC-MS for oligonucleotide analysis

We used a Waters microflow C18 column in our oligonucleotide analysis. We found that the concentrations of TEA and HFIP are important to achieve a good chromatography peak for the microflow column. The LC mobile phases with the ion-pairing reagent need to be freshly prepared and typically used within a day for direct sensitivity comparison. **Figure 1A** shows the representative extracted ion chromatogram of the most abundant charge state (10-) from each platform. We observed a slight charge envelope shift towards lower m/z (higher charge states) for MnESI compared to high-flow HESI (**Figure 1B**). In addition, majority of the MS signals from MnESI were from the three higher charge states (9- to 11-), suggesting better and more homogenous ionization of oligo DNA for microflow LC-MS. By either preconditioning the microflow column with EDTA or preparing oligonucleotide sample in 1 mM TDTA, we found the salt adducts (Na+, K+) were almost completely removed, confirming the MnESI workflow suitable for accurate quantification of oligonucleotides.

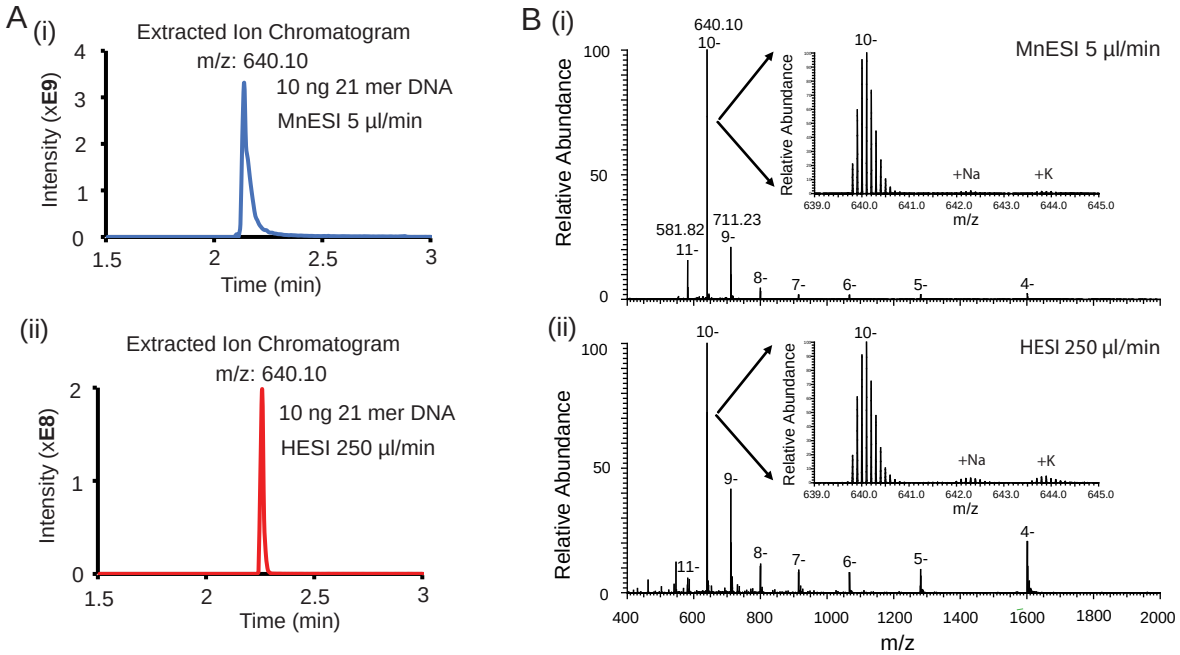

Figure 1. LC-MS analysis of a 21mer oligonucleotide using both MnESI and high-flow HESI. **A.** Representative extracted ion chromatograms of the most abundant charge state from the two platforms. **B.** Full mass spectrum at m/z 400-2000 for MnESI (i) and high-flow HESI (ii). Compared to high-flow HESI, MnESI shows a charge envelope that is concentrated towards the higher m/z region (9- to 11-). The zoom-in views show the isotopic envelope of the 10- charge state and low salt adducts.

2. MnESI-MS platform significantly improves sensitivity for oligonucleotide analysis

We examined the sensitivity gain of MnESI at 5 µl/min over the conventional HESI platform at 250 µl/min using 10 ng on-column DNA oligo under the optimized conditions for each analysis.

Two quantification methods were used for comparison including the peak area of the extracted ion chromatogram of the top 3 charge states (9- to 11-) and using the charge deconvolution method (4- to 11-). We achieved significant and consistent sensitivity gain from MnESI over high-flow HESI, specifically an average of 37.8-fold using the EIC peak area (**Figure 2A**) and 44-fold using the charge deconvolution (**Figure 2B**). In addition, we found very good reproducibility from both platforms with their CVs of less than 5%.

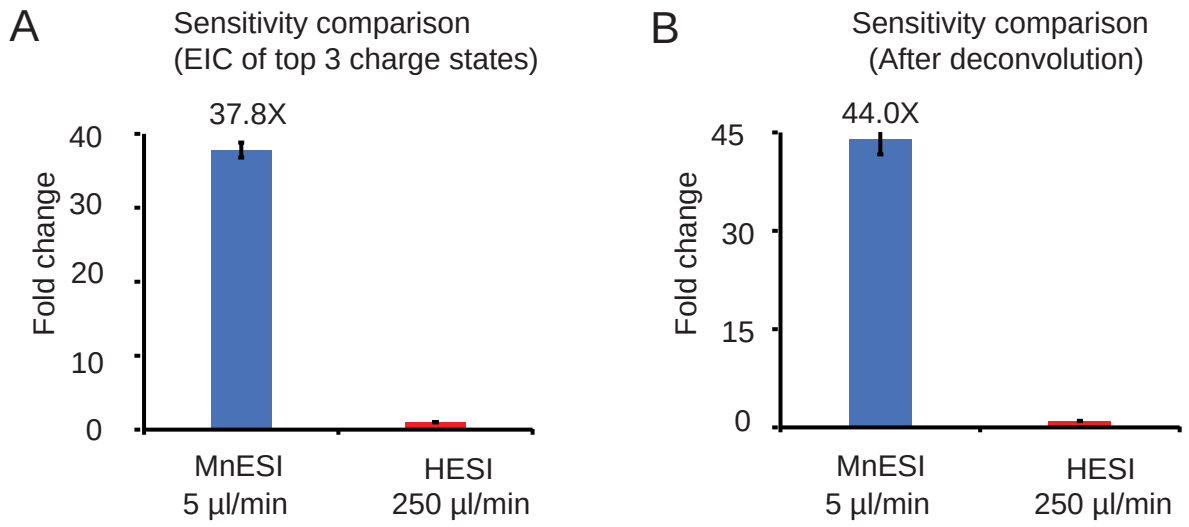

Figure 2. Sensitivity comparison of MnESI and high-flow HESI for oligonucleotide quantitation. **A**. Sensitivity comparison using the peak area intensity from extracted ion chromatogram of the top 3 charge states (9- to 11-). **B**. Sensitivity comparison using the charge deconvolution method (4- to 11-).

3. MnESI-MS platform seamlessly integrates with the high-flow LC system for oligonucleotide analysis

Since the traditional workflow for oligonucleotide analysis is high-flow LC-MS by HESI, we evaluated the performance of coupling MnESI with a high-flow LC system and 2.1 mm ID column using a Newomics® post-column splitting kit (**Figure 3A**). A T-splitter was used to split the analytical flow of 250 µl/min from the LC column down to a microflow of 12.5 µl/min (1:20 splitting), and delivered to a 10 µm-ID, 8-nozzle M3 emitter for spray into MS. A representative extracted ion chromatogram of the most abundant charge state (10-) is shown in **Figure 3B**. Compared to the conventional high-flow method without the post-column splitting, this new method by flow splitting to M3 emitters achieved slightly better sensitivity (~1.6 fold), even though only 5% of oligonucleotide was delivered to MS (**Figure 3C**). Our MnESI-MS workflow with high-flow post-column splitting allows the application of MnESI for microflow without the need to change the high-flow LC system. In addition, majority of the ion-pairing reagent from the high-flow column was diverted to waste rather than going into the mass spectrometer, minimizing the contamination of ion cone and C-trap of the QE instrument. This will significantly improve the assay robustness and instrument stability for oligonucleotide quantitation.

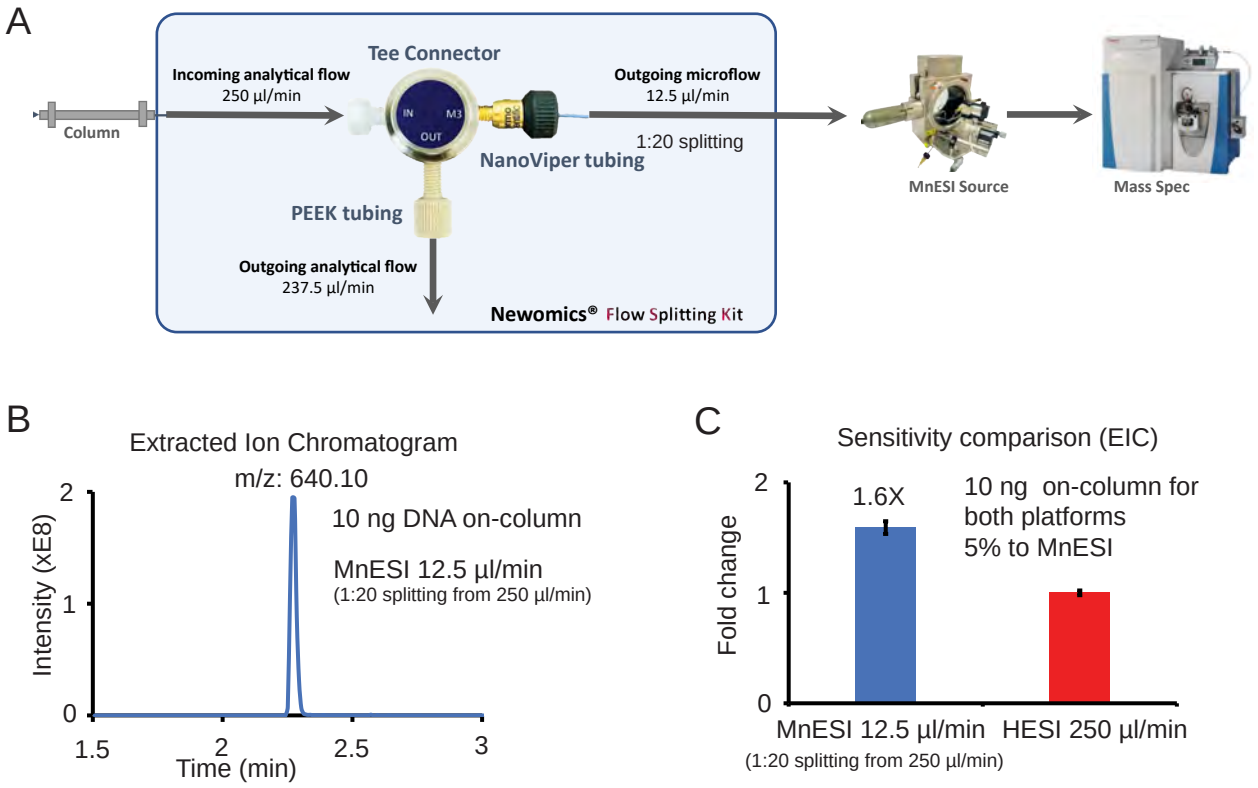

Figure 3. Integration of MnESI-MS platform with the high-flow LC-MS workflow using a post-column splitting kit. **A.** Workflow using a Newomics flow splitting kit. **B**. Representative extracted ion chromatogram of the most abundant charge state (10-) from LC-MnESI-MS at 12.5 µl/min (after 1:20 splitting from 250 µl/min). **C**. Sensitivity comparison of LC-MnESI-MS at 12.5 µl/min (after 1:20 splitting from 250 µl/min) and HESI at 250 µl/min (high-flow LC-MS). The same amount of starting oligonucleotide (10 ng on-column) was injected for both workflows. A 10 µm- ID, 8-nozzle M3 emitter was used in the analysis for MnESI-MS.

Conclusion

We have established a new MnESI-MS platform for sensitive LC-MS analysis of oligonucleotides. By interfacing to an Orbitrap Q Exactive Plus mass spectrometer, Newomics® MnESI platform has achieved high sensitivity for analysis of oligonucleotides. We demonstrate the following significant advantages of our LC-MnESI-MS platform using M3 emitters over the traditional high-flow LC-MS platform using HESI:

1. More than **30-fold** sensitivity gain using the microflow LC-MS at the 5 ul/min flow rate.
2. Sensitivity increased 1.6-fold even after 1:20 flow splitting of the high flow, with only 5% material entering the MS.
3. Flow splitting reduced contamination due to much less ion-paring reagent entering the MS.

Reference

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6. Newomics Application Notes: https://www.newomics.com/applications/m3-emitters/

Ordering Information

Product	Catalog #
MnESI Source for Thermo Scientific New Generation Mass Spectrometers	IS-T01
MnESI Source for Thermo Scientific Legacy Mass Spectrometers	IS-T02
MnESI Source for Bruker Mass Spectrometers	IS-B01
M3 Emitter, 10 µm ID, 8-nozzle	E8N10MU01
M3 Emitter, 20 µm ID, 8-nozzle	E8N20MU01
M3 Emitter, 20 µm ID, 5-nozzle	E5N20MU01
Flow Splitting Kit for Microflow LC-MS, 1-5 µl/min	FSK-01
Flow Splitting Kit for Microflow LC-MS, 5-15 µl/min	FSK-02
Flow Splitting Kit for Microflow LC-MS, 10-25 µl/min	FSK-03
Flow Splitting Kit for Microflow LC-MS, custom	FSK-10

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